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Albumin Permeability Across Endothelial Cell Monolayer Exposed to Reactive Oxygen Intermediates: Involvement of Reversible Functional Alteration of the Cell Membrane Ca^{2+} Channels

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ABSTRACT

This study was designed to test the idea that the redox state of sulfhydryl (SH)-groups in cell-membrane Ca^{2+} channels plays a pivotal role in Ca^{2+} influx, which in turn causes an increase in albumin permeability across the cultured monolayer of porcine pulmonary artery endothelial (PPAE) cells exposed to xanthine/xanthine oxidase (X/XO). Albumin permeability as well as the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was increased by X/XO. A H_2O_2 scavenger (catalase), an iron chelator (o-phenanthroline), and a hydroxyl radical scavenger (dimethyl sulfoxide) inhibited these changes provoked by X/XO, in which intracellular iron-catalyzed hydroxyl radical generation was suggested to be involved. The increase in albumin permeability and $[Ca^{2+}]_i$ continued once the PPAE cells were exposed to X/XO. The $[Ca^{2+}]_i$ was decreased by a Ca^{2+} channel blocker, Ni^{2+}, while the removal of Ni^{2+} increased $[Ca^{2+}]_i$ again, suggesting the sustained Ca^{2+} influx through cell-membrane Ca^{2+} channels was responsible for the $[Ca^{2+}]_i$ elevation. Ni^{2+} failed to inhibit albumin permeability sustained after the removal of X/XO. In contrast, SH-reducing agents (dithiothreitol and glutathione) inhibited the sustained permeability as well as Ca^{2+} influx. We concluded that the redox alteration of SH-groups in cell-membrane Ca^{2+} channels was involved in the increase in albumin permeability after exposure of the endothelial cells to oxidative stress.

Key words: *Albumin permeability, Reactive oxygen intermediates, Calcium channels, Redox state*

Endothelial cells cover the internal surface of blood vessels and regulate the permeability of various solutes. Impairment of endothelial permeability, especially to albumin, has been implicated in several life-threatening events such as pulmonary edema associated with neutrophil-mediated lung injury[8,12,18,21)]. It is known that reactive oxygen intermediates (ROIs) yielded and released from activated neutrophils, i.e., superoxide anion ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2), are involved in the increased albumin permeability during such pathophysiological conditions[8,12,21)].

An enzymic, thus, exogenous oxidant generating system, xanthine and xanthine oxidase (X/XO), has been used as a reliable tool to assess the precise mechanisms of ROIs-induced change in albumin permeability across the endothelial cell monolayer[4,19)]. Shasby et al reported that the enhanced albumin permeability continues after the exposure of endothelial cells to ROIs, but can be suppressed by the treatment of these cells with a medium containing fetal calf serum[19)]. These findings suggested that the increase in albumin permeability is functional, but is not caused by lethal endothelial cell damage by ROIs. However, the exact component(s) of such a medium that regulates endothelial permeability to albumin remains to be established.

It is known that vascular permeability is regulated as a function of the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) through causing gaps between endothelial cells stimulated with several inflammatory agonists, e.g., histamine or thrombin[13,14,16)]. Sublethal concentrations of ROIs have also been reported to increase $[Ca^{2+}]_i$: a growing body of evidence from a number of types of cells indicates that Ca^{2+} influx through cell-membrane Ca^{2+} channels is involved in the $[Ca^{2+}]_i$ elevation provoked by ROIs[3,5–7,11,17)]. Recent studies including ours further suggested that the redox state of sulfhydryl (SH)-groups in these channels plays a pivotal role in the activation of Ca^{2+} influx[3,5,11,17)].

Therefore, the present study was designed to test the idea that the redox state of SH-groups is also involved in ROIs-induced change in albumin permeability across the endothelial cell monolayer. The reversibility of sustained albumin permeability by treatment with SH reducing agents was also discussed.

MATERIALS AND METHODS

Endothelial cell culture

Isolation and primary culture of porcine pulmonary artery endothelial (PPAE) cells were performed according to the method previously described for aortic endothelial cells with modification[1,2]. In brief, pulmonary arteries obtained at a local slaughterhouse were rinsed with ampicillin/kanamycin containing (45 μg/ml, both) Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS) immediately after the animals were sacrificed. These arteries were transferred to our laboratory and incubated at 25°C for 20 min with trypsin (0.125%) in EDTA (0.02%)-containing PBS. PPAE cells were then harvested by stroking the inner surface of the arteries gently with a scalpel blade and were suspended in RD medium [1:1 (v/v) RPMI 1640 medium/Dulbecco's modified Eagle's medium (DMEM)] supplemented with bicarbonate (2 mg/ml), HEPES (15 mM), ampicillin (90 μg/ml), kanamycin (90 μg/ml), and 10% (v/v) fetal bovine serum (FBS). These cells were grown in 25 cm^2 collagen-coated plastic flasks and cryopreserved in FBS containing 10% (v/v) dimethyl sulfoxide (DMSO) at a density of 1–2 × 10^6 cells/ml under liquid nitrogen after the confluence of passage 2 (split ratio, 1:3). The endothelial identity of these cells was confirmed post-cryopreservation by the uptake of di-iodoacetyl-low-density lipoprotein using fluorescence microscopy[7]. The method confirmed that > 99% of these cells consisted of endothelial cells.

Measurement of albumin permeability across PPAE monolayer

Passage 3–4 PPAE cells were seeded at a 1:3 split ratio on collagen-coated micropore filters (pore size, 0.4 μm) attached to the inner chamber of a Costar Transwell (0.33 cm^2/well) and cultured until the cells reached to a confluent monolayer. The confluence of PPAE monolayers used in this study was confirmed by close observation under a phase contrast microscope. The cell attached chambers were placed into 24-well cluster dishes filled with 800 μl of modified Hanks balanced salt solution (HBSS), while the inner chamber (i.e., luminal side of PPAE monolayer) was filled with 200 μl of the same buffer except that it contained fluorescein isothiocyanate (FITC)-albumin (5 mg/ml). These volumes were determined to avoid a hydrostatic pressure gradient across the filter. The composition of HBSS was (in mM): NaCl, 138; KCl, 4.7; $CaCl_2$, 1.3; $MgSO_4$, 0.8; KH_2PO_4, 0.4; K_2HPO_4, 0.3; D-glucose, 5.6; HEPES, 4.2; and diethylenetriaminepentaacetic acid (DTPA), 0.02 (pH 7.4). HBSS contained the iron chelator (DTPA) at a minimum but significant concentration to eliminate the exogenous transition metal-dependent production of hydroxyl radical from commercially available xanthine oxidase[2,3]. Various drugs including xanthine, xanthine oxidase, and ROIs inhibitors were added to the inner chamber. The cluster dishes were continuously shaken at 37°C by using an orbital shaker (Iuchi, Osaka, Japan) at 60 r.p.m. under a humidified atmosphere in air. The buffer of each dish (i.e., abluminal side of PPAE monolayer) was collected to determine albumin permeability, defined as the concentration of FITC-albumin transferred across the PPAE monolayer during pre-selected incubation periods. The fluorescence intensity of FITC-albumin in each sample was measured by using a fluorescence spectrophotometer (èxcitation, 495 nm; emission, 510 nm) (URF-1000, Shimadzu, Kyoto, Japan). The concentration of FITC-albumin was determined by fitting the fluorescence intensity to a calibration curve obtained from standard FITC-albumin solutions prepared just before the experiment.

Measurement of $[Ca^{2+}]_i$

PPAE cells split from the same origin were cultured on a fibronectin-coated glass coverslip (45 × 45 mm), attached to silicon rubber septa separated into four 15 mmø chambers. $[Ca^{2+}]_i$ of PPAE cells was measured using a fluorescent Ca^{2+} indicator dye, fura-2, exactly according to the method described previously[3]. The coverslip was placed on the stage of a fluorescence inverted microscope, combined with a computer Ca^{2+}-analyzing system (ARGUS-50/CA2, Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence intensity with excitation at 340/380 nm and emission at 510 nm was recorded every 30 sec under continuous perfusion of HBSS (1 ml/min, 37 °C) containing various agents. The ratio of fluorescence intensity was converted to $[Ca^{2+}]_i$ by using an in-vitro calibration curve obtained from standard Ca^{2+}/EGTA solutions containing 5 μM fura-2 free acid. The mean value of $[Ca^{2+}]_i$ obtained from a randomly selected 21 cells in a microscopic field was considered as the $[Ca^{2+}]_i$ in each experiment.

Exogenous oxidant generating system

Production of $^{\bullet}O_2^-$ and H_2O_2 was achieved by adding 0.04 U/ml xanthine oxidase to HBSS in the presence of xanthine at a final concentration of 200 μM. In a preliminary study, we observed that the fluorescence intensity of FITC-albumin was increased by the addition of xanthine oxidase. This was caused by the degradation of FITC-albumin, possibly due to a contamination with trypsin of

commercially available xanthine oxidase[15]. Therefore, we added a serine protease inhibitor, phenylmethylsulfonyl fluoride, at a final concentration of 1 mM to Sigma grade-I xanthine oxidase. We confirmed by gel chromatography that no degradation of FITC-albumin occurs in this experimental setting. The amount of xanthine oxidase in HBSS was adjusted before each batch of experiments using a spectrophotometer (DU 640, Beckman, Fullerton, CA) as previously described[2,3].

Identification of cell-membrane damage

The PPAE monolayers exposed to X/XO were incubated with a hydrophilic fluorescent dye, propidium iodide (PI; 1 μg/ml), to examine the breakage of the cell membrane, which can be detected by the fluorescence of PI at the nuclei of PPAE cells[10]. The number of PI-stained cells was counted by using a fluorescence microscope combined with a digital image analysis system (ARGUS-50, Hamamatsu Photonics). PPAE cells treated with or without 0.1% triton-X were used as a positive and a negative control, respectively.

Materials

Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was obtained from Nunc (Brooklyn, NSW, Australia). Trypsin, superoxide dismutase (SOD), catalase, xanthine, xanthine oxidase, glutathione (GSH), diethylenetriaminepentaacetic acid (DTPA), allopurinol, fluorescein isothiocyanate (FITC)-albumin, N-[2-hydroxyethyl] piperazine-N'-[2 ethanesulfonic acid] (HEPES), phenylmethysulfonyl fluoride, ampicillin, and kanamycin were purchased from Sigma (St. Louis, MO, U.S.A.). Ethylene-diaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), and o-phenanthroline were from Katayama (Osaka, Japan). Acetoxymethyl ester form of 1, 2-bis (o-amynophenoxy) ethane-N, N, N, 'N'-tetraacetic acid (BAPTA/AM) and of fura-2 (fura-2/AM) were purchased from Dojindo Laboratories (Kumamoto, Japan). Transwell filters were obtained from Costar (Cambridge, MA, U.S.A.). Di-iodoacetyl-low-density lipoprotein was from Funakoshi (Tokyo, Japan). All other chemicals were of analytical quality.

Data analysis

Data were expressed as mean ± SE. Differences between groups were analyzed for statistical significance using analysis of variance followed by Bonferroni's test. A P value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Mimicking action of albumin permeability to cytosolic Ca^{2+} movement of PPAE cells exposed to X/XO

The basal albumin permeability was 43 ± 8.8 μg/ml/30 min, while a 30 min exposure of the monolayer to 200 μM xanthine and 0.04 U/ml xanthine oxidase (X/XO) increased permeability to 86 ± 26 μg/ml/30 min (n=6, respectively) (for the protocol, see Fig. 1A). In monolayers exposed to X/XO, endothelial retraction and intercellular gaps were observed under a phase contrast microscope after silver nitrate staining (not shown). Exposure of these cells to X/XO for at least 120 min did not influence the number of PI-stained cells (basal: 2.3 ± 0.5% and X/XO: 3.1 ± 0.6%, respectively; n=6), confirming that the increase in albumin permeability was not caused by X/XO-provoked lethal cell-membrane damage. Addition of superoxide dismutase (SOD; 300 U/ml) failed to influence the X/XO-mediated increase in albumin permeability, while a H_2O_2 scavenger, catalase (300 U/ml), significantly inhibited the permeability during the

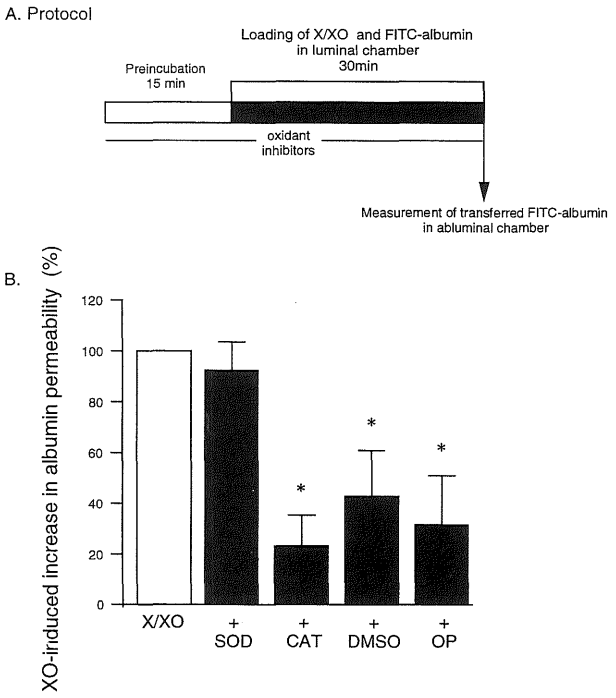

Fig. 1. Effects of oxidant inhibitors on albumin permeability across cultured monolayer of porcine pulmonary artery endothelial (PPAE) cells exposed to 200 μM xanthine and 0.04 U/ml xanthine oxidase (X/XO). (A) Experimental protocol. Albumin permeability was examined as the concentration of 5 mg/ml (FITC)-albumin transferred during the 30-min exposure of PPAE cells to X/XO at 37 °C. (B) Percent change, assuming albumin permeability in the absence or presence of X/XO exposure to be 0% and 100%, respectively. Data are expressed as mean ± SE of 4–6 separate experiments. PPAE cells used were obtained from at least three separate cell lines. SOD, superoxide dismutase (300 U/ml); CAT, catalase (300 U/ml); DMSO, dimethyl sulfoxide (70 mM); OP, o-phenanthroline (100 μM). *Significant difference from X/XO ($p < 0.05$).

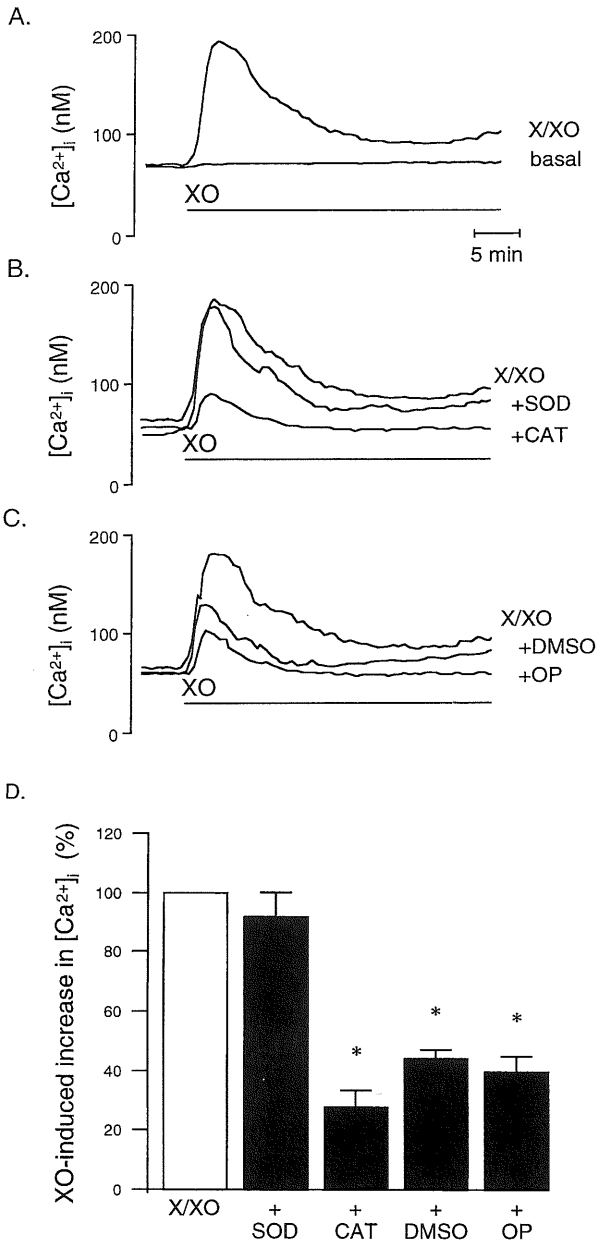

Fig. 2. Effects of oxidant inhibitors on cytosolic Ca^{2+} movement of porcine pulmonary artery endothelial (PPAE) cells exposed to 200 μM xanthine and 0.04 U/ml xanthine oxidase (X/XO). Intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) was measured at 37°C by using Fura-2 fluorometry. (A) representative recordings of $[Ca^{2+}]_i$ in PPAE cells in the absence or presence of X/XO. (B) Superoxide dismutase (SOD, 300 U/ml) or catalase (CAT, 300 U/ml) was added at 15 min prior-and during the exposure of PPAE cells to X/XO. (C) Dimethyl sulfoxide (DMSO, 70 mM) or o-phenanthroline (OP, 100 μM) was added at 15 min prior-and during the X/XO exposure. (D) Percent change in maximum $[Ca^{2+}]_i$ of PPAE cells, assuming the basal $[Ca^{2+}]_i$ and X/XO-provoked peak $[Ca^{2+}]_i$ in the absence of oxidant inhibitors to be 0% and 100%, respectively. Data are expressed as mean ± SE of 4–6 separate experiments. PPAE cells used were obtained from at least three separate cell lines. SOD, superoxide dismutase (300 U/ml); CAT, catalase (300 U/ml); DMSO, dimethyl sulfoxide (70 mM); OP, o-phenanthroline (100 μM). *Significant difference from X/XO ($p < 0.05$).

X/XO exposure (Fig. 1B). However, we confirmed that albumin permeability did not increase by a 30 min exposure of the PPAE monolayers to 200 μM H_2O_2, the amount of which was equal to that yielded from 200 μM xanthine (n=4). It is unlikely, therefore, that H_2O_2 was solely responsible for the increase in albumin permeability by X/XO exposure. A 10 min pre-and co-incubation of PPAE cells with a cell-membrane permeable iron chelator, o-phenanthroline (100 μM), or a hydroxyl radical ($^{\bullet}OH$) scavenger, DMSO (70 mM), prior to and during X/XO exposure also inhibited the albumin permeability (Fig. 1B), implicating the involvement of intracellular iron-dependent $^{\bullet}OH$ generation. To assess whether the cytosolic Ca^{2+} movement in PPAE cells exposed to X/XO explains the change in albumin permeability, we then measured $[Ca^{2+}]_i$ by using fura-2 fluorometry. The exposure of PPAE cells to X/XO promptly increased $[Ca^{2+}]_i$. During the X/XO exposure, $[Ca^{2+}]_i$ was elevated to a maximum level, then gradually declined to levels higher than that observed before the exposure (Fig. 2A). Because a single application of 200 μM xanthine or 0.04 U/ml xanthine oxidase failed to increase $[Ca^{2+}]_i$ (not shown, n=4), ROIs yielded from X/XO appeared to be responsible for the $[Ca^{2+}]_i$ elevation in PPAE cells. We confirmed that the addition of catalase, o-phenanthroline, and DMSO significantly inhibited the X/XO-provoked $[Ca^{2+}]_i$ elevation, while SOD failed to influence the Ca^{2+} movement (Fig. 2B-D). The rank order of efficacy of these ROIs inhibitors for X/XO-provoked Ca^{2+} movement mimicked that for albumin permeability.

Differential effects of a Ca^{2+} channel blocker and an intracellular Ca^{2+} chelator on albumin permeability of PPAE monolayer exposed to X/XO

As shown in Fig. 3A, the increase in $[Ca^{2+}]_i$ provoked by X/XO continued after the removal of X/XO. In this set of experiments, therefore, we evaluated the effects of a Ca^{2+} channel blocker, Ni^{2+}, and an intracellular Ca^{2+} chelator, BAPTA/AM, on albumin permeability as well as on cytosolic Ca^{2+} movement during and after the exposure to X/XO (for the protocol, see Fig. 3B). In the presence of Ni^{2+}, $[Ca^{2+}]_i$ decreased to basal levels after a transient $[Ca^{2+}]_i$ elevation. Such initial transient $[Ca^{2+}]_i$ elevation appeared to be caused by Ca^{2+} release from intracellular sources because preincubation of the cells with 300 μM thapsigargin, which is known to deplete intracellular Ca^{2+} stores, completely suppressed the $[Ca^{2+}]_i$ transient (not shown, n=3). The peak $[Ca^{2+}]_i$ of the initial transient was significantly smaller than that in the absence of Ni^{2+}. These findings suggested that Ca^{2+} influx through cell-membrane Ca^{2+} channels during and following Ca^{2+} release from intracellular stores was the main source of X/XO-provoked

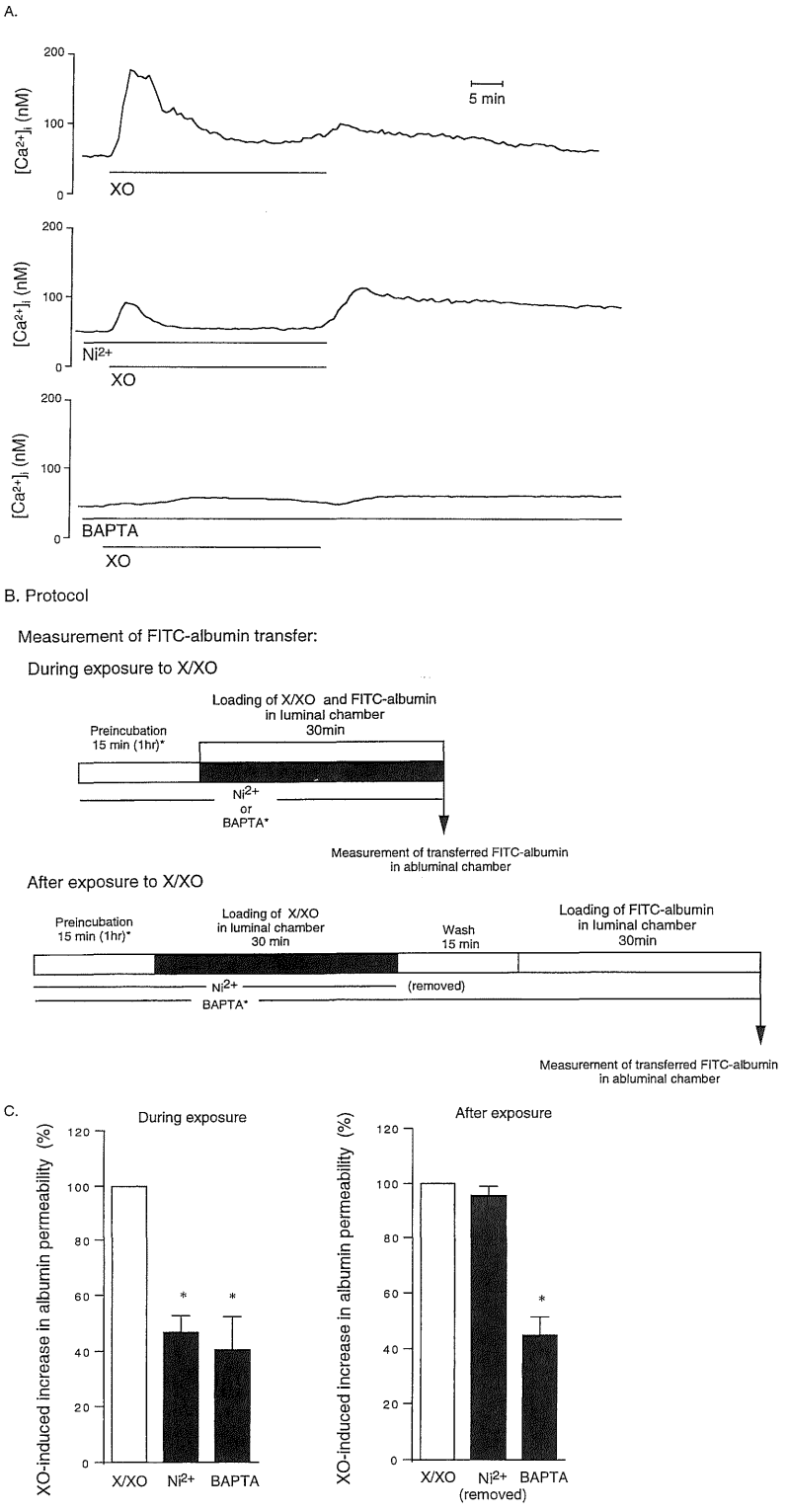

Fig. 3. (A) Representative recordings of cytosolic Ca^{2+} movement of porcine pulmonary artery endothelial (PPAE) cells during and after the exposure to 200 μM xanthine and 0.04 U/ml xanthine oxidase (X/XO). Intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) was measured at 37°C by using Fura-2 fluoremetry. Upper trace: X/XO was removed after a 30-min exposure. Middle trace: same condition as in the upper trace except that PPAE cells were exposed to X/XO in the presence of 1.5 mM Ni^{2+}. Ni^{2+} was removed together with X/XO. Lower trace: same condition as in the upper trace except that PPAE cells were pre-loaded with 5 μM BAPTA/AM. (B) Experimental protocol to examine the albumin permeability, examined as the concentration of 5 mg/ml (FITC)-albumin transferred: (1) during exposure of PPAE cells to X/XO for 30 min at 37 °C; and (2) during the 30-min incubation period following the 30-min X/XO exposure. *Preincubation time for BAPTA/AM was 1 hour. (C) Percent change, assuming albumin permeability in the absence or presence of X/XO exposure to be 0% and 100%, respectively. Data are expressed as mean ± SE of 4–6 separate experiments. PPAE cells used were obtained from at least three separate cell lines. *Significant difference from X/XO ($p < 0.05$).

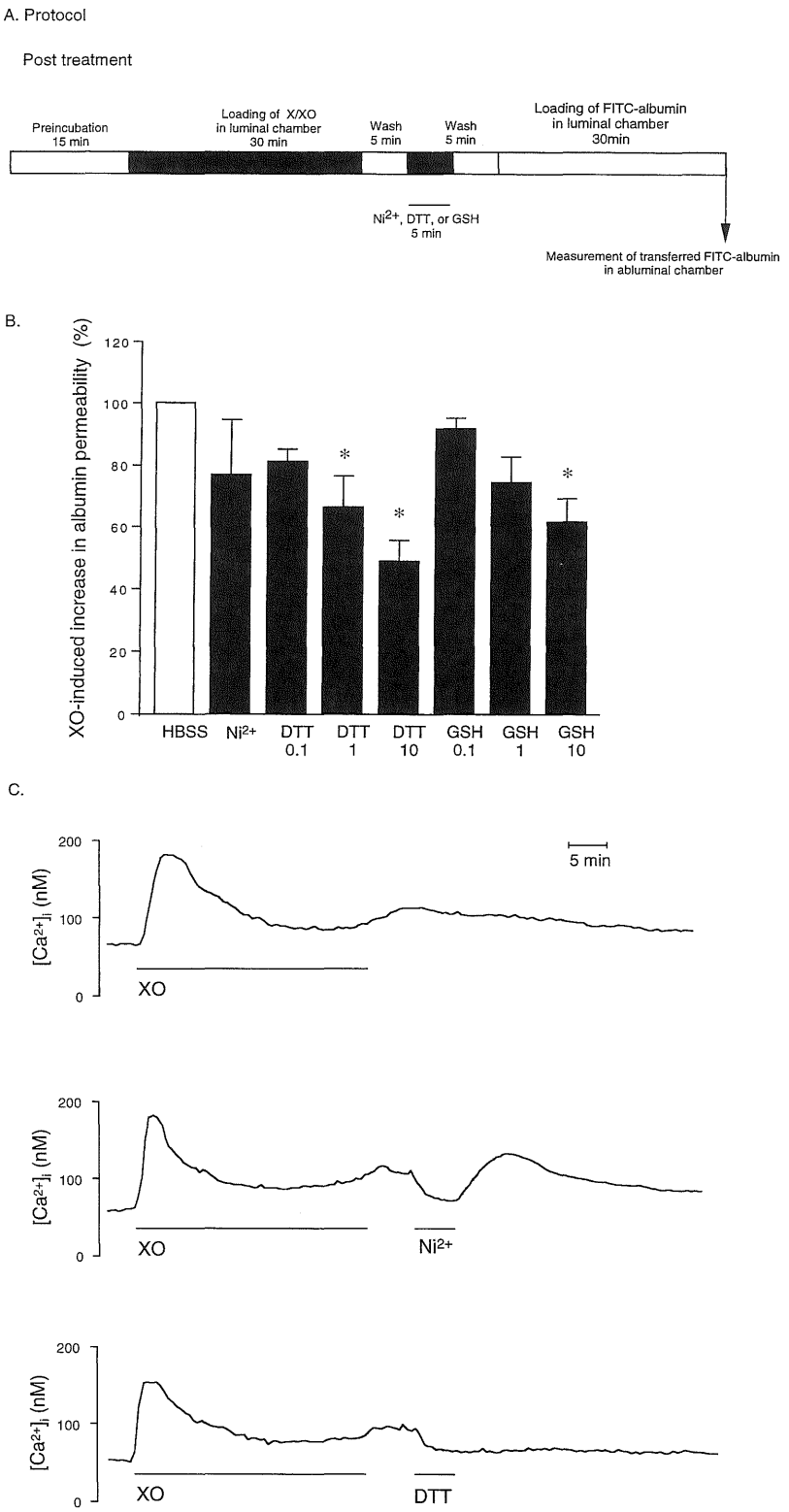

Fig. 4. Effects of sulfhydryl (SH)-reducing agents and Ni^{2+} on albumin permeability across cultured monolayer of porcine pulmonary artery endothelial (PPAE) cells exposed to 200 μM xanthine and 0.04 U/ml xanthine oxidase (X/XO). (A) experimental protocol. Ni^{2+} (1.5 mM), dithiothreitol (DTT, 0.1–10 mM), or reduced form of glutathione (GSH, 0.1–10 mM) was added to PPAE cells after a 30-min exposure to X/XO. (B) Percent change, assuming albumin permeability in the absence or the presence of X/XO exposure to be 0% and 100%, respectively. Data are expressed as mean ± SE of 4–6 separate experiments. PPAE cells used were obtained from at least three separate cell lines. *Significant difference from X/XO ($p < 0.05$). (C) Representative recordings of cytosolic Ca^{2+} movement in PPAE cells. Intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) was measured at 37°C by using Fura-2 fluorometry. Upper trace: PPAE cells were exposed to X/XO for 30 min. Middle trace: Ni^{2+} was added to PPAE cells for 5 min after the 30-min exposure of the cells to X/XO. Lower trace: same condition as in the middle trace except that DTT was added instead of Ni^{2+}.

$[Ca^{2+}]_i$ elevation. The removal of Ni^{2+} promptly elevated $[Ca^{2+}]_i$. In contrast, $[Ca^{2+}]_i$ of PPAE cells preincubated with BAPTA/AM, which entered the cells and became an intracellular Ca^{2+} chelator (BAPTA), did not significantly change throughout the observation (> 1.5 hr). Taking these findings together, it is likely that the continuous $[Ca^{2+}]_i$ elevation after removal of X/XO was due to the sustained Ca^{2+} influx through cell-membrane Ca^{2+} channels.

We also observed that Ni^{2+} inhibited albumin permeability only during X/XO exposure, i.e., Ni^{2+} failed to inhibit the increase in permeability that continued after X/XO were removed. In contrast, BAPTA/AM inhibited the permeability both during and after X/XO exposure (Fig. 3C). These findings suggested that the continuous Ca^{2+} influx through the cell-membrane Ca^{2+} channels was involved in the enhanced albumin permeability sustained after the X/XO exposure.

Effect of SH reducing agents on sustained albumin permeability after X/XO exposure

We have recently reported that the redox state of SH-groups in Ca^{2+} channels was important for the activation of cell membrane Ca^{2+} channels in endothelial cells[3]. Therefore, we examined the effect of SH-reducing agents, which were applied after the exposure of PPAE monolayers to X/XO, on albumin permeability in comparison with that of Ni^{2+}. These agents were transiently added to the PPAE monolayers for 5 min after the removal of X/XO (for the protocol, see Fig. 4A). As suggested in the previous section, Ni^{2+} did not inhibit the sustained albumin permeability after X/XO exposure (Fig. 4B). The fura-2 fluorometry revealed that Ni^{2+} could decrease $[Ca^{2+}]_i$ only during the existence of this Ca^{2+} channel blocker (Fig. 4C). In contrast, a 5 min loading of PPAE monolayers to DTT (≥ 1 mM) or GSH (≥ 10 mM) suppressed the sustained increase in albumin permeability (Fig. 4B). We also confirmed that the decrease in $[Ca^{2+}]$ following the addition of these SH-reducing agents continued after the removal of these agents (Fig. 4C). These results suggested that the redox state of SH groups in the Ca^{2+} channels regulated the sustained albumin permeability after X/XO exposure.

DISCUSSION

In the present study, we demonstrated that the change in albumin permeability across a cultured endothelial cell monolayer exposed to an oxidant generator, X/XO, was strongly associated with cytosolic Ca^{2+} movement in these cells. Although the direct products of X/XO was $^{\bullet}O_2^-$ and H_2O_2[2], we suggested that a further reduced oxygen metabolite, $^{\bullet}OH$, generated through the intracellular transition metal(s)-dependent pathway (Haber-Weiss cycle) was also involved in albumin permeability as well as cytosolic Ca^{2+} movement. This conclusion is based on the finding that a cell membrane permeable iron chelator (o-phenanthroline) and a $^{\bullet}OH$ scavenger (DMSO) inhibited the changes in $[Ca^{2+}]_i$ and albumin permeability. Although we confirmed that the addition of SOD in combination with catalase abolished the increase in both $[Ca^{2+}]_i$ and albumin permeability (data not shown), a single administration of SOD did not inhibit these X/XO-provoked changes. As we previously reported, the enhanced Fe^{+3}-dependent $^{\bullet}OH$ generation from X/XO through SOD accelerated H_2O_2 production may explain our results in this study[2,3].

An early series of experiments from Zweier et al[22-24] and ours[2] demonstrated that intracellular generation of $^{\bullet}OH$ is involved in X/XO provoked endothelial cell injury. A recent study of ours further demonstrated that the cytotoxicty of X/XO in endothelial cells, examined as the inhibition of cellular respiratory function, is triggered by the increase in $[Ca^{2+}]_i$, the main source of which is Ca^{2+} influx through cell-membrane Ca^{2+} channels[3]. In this report, the redox alteration of SH-groups in these Ca^{2+} channels is potently involved in the Ca^{2+} influx which continued even after the removal of X/XO. It also showed that SH-reducing agents suppressed such prolonged Ca^{2+} influx. Similar results have been reported from other investigators using canine venous endothelial cells[7] or A7r5 rat aortic smooth muscle cells[17] exposed to H_2O_2. These previous findings are consistent with our opinion. We assumed the mechanism(s) of this phenomenon as follows: X/XO induces Ca^{2+} influx by activation of Ca^{2+} channels, and the activated condition continues after removal of X/XO. However, it can be inhibited when Ni^{2+} is present. After removal of Ni^{2+} in this condition, the Ca^{2+} channels can apparently become activated and can convey Ca^{2+} to increase $[Ca^{2+}]_i$. When DTT or GSH are administered instead of Ni^{2+}, they can hinder the activation of the channels even after their removal since they produce conformational changes in the channels. An increase in $[Ca^{2+}]_i$ induces endothelial cell retraction and intercellular gaps to disrupt the integrity of the endothelial cell monolayer. Namely, Ca^{2+} influx through cell-membrane Ca^{2+} channels after exposure to X/XO also plays a pivotal role in the prolonged albumin permeability across the endothelial monolayer exposed to X/XO.

The most important point in the current study is that the extracellular application of DTT or GSH reversed the albumin permeability, sustained after the ROIs exposure. The latter agent is clinically applicable to subjects in several pathophysiological situations relating to ROIs-mediated lung injury. Therefore, it is interesting to note that the addition of DTT or GSH promptly decreased the X/XO-provoked albumin permeabili-

ty and $[Ca^{2+}]_i$, although the concentration of these agents effectively applied to endothelial cells was as high as 1–10 mM. The actual plasma concentration of GSH when administrated as a bolus infusion through mixed venous remains to be established. Because we confirmed that thiol reductants administered with X/XO also suppressed the increase in albumin permeability and $[Ca^{2+}]_i$ (not shown), these reagents may exert inhibitory effects as well as in clinical settings in which thiol reductants may directly react with oxidants.

In spite of the significance of cytosolic Ca^{2+} movement observed in ROIs provoked change in albumin permeability, other unknown mechanism (s) are thought to be involved because of the incomplete inhibition by Ni^{2+} or BAPTA/AM of the X/XO-induced increase in albumin permeability. Activation of protein kinase C (PKC) has been implicated in the modulation of endothelial permeability through the development of "sustained" endothelial cell contraction following $[Ca^{2+}]_i$ elevation. It is known that a direct activation of PKC increases the albumin permeability that is independent of Ca^{2+} gating mechanisms. This explains why PKC plays a proximal role in endothelial permeability[9]. It has been reported that the inhibition of PKC suppresses the increase in albumin permeability across the endothelial cell monolayer exposed to H_2O_2[20], while the direct action of ROIs to activate PKC has not yet been clarified.

In conclusion, the present study demonstrated that the exposure of endothelial cells to X/XO induced a sustained Ca^{2+} influx through cell-membrane Ca^{2+} channels, which in turn caused an increase in albumin permeability. The persistent increase in $[Ca^{2+}]_i$ and albumin permeability were suppressed by SH reducing agents, indicating that the change in the redox state of SH-groups of Ca^{2+} channels are likely to be involved in this process.

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